Cytokine signaling: **Cytokine-inducible signaling inhibitors**

M. Javad Aman and Warren J. Leonard

Seven members of a family of cytokine-inducible proteins have been identified, a number of which have been shown to act as negative regulators of cytokine action. All of these proteins contain a central Src homology 2 domain, as well as conserved motifs in their carboxy-terminal regions.

Address: Laboratory of Molecular Immunology, National Institute of Health, National Heart, Lung, and Blood Institute, Bethesda, Maryland 20893, USA.

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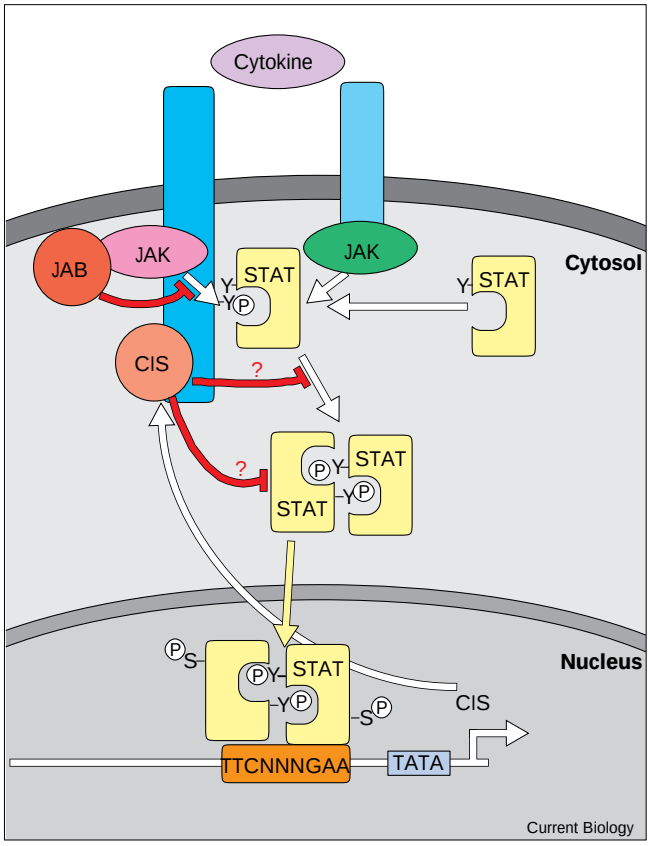
Cytokines are a broad array of molecules that mediate signals important for many biological functions, including growth, differentiation, and apoptosis. Cytokines can exert actions on the cells that produce them (autocrine signals) or on other cells (paracrine signals). Type I cytokines have similar four α-helical bundle structures and are also referred to as 'up–up–down–down' helical cytokines because of the topological orientation of the sequential α helices. In addition to the similarity in structure of type I cytokines, the receptors of these molecules form a family and share certain signaling pathways, most notably the pathway involving Janus kinases (JAKs) and signal transducers and activators of transcription (STATs). The group of type II cytokines includes both interferon (IFN)-α/β and IFN-γ as well as interleukin 10 (IL-10). These cytokines have different structures from the type I cytokines, but they also exert their actions, at least in part, via the JAK–STAT pathway.

Over the years, the majority of investigations on cytokines have focused on the mechanisms by which they exert their actions. It is clear, however, that the actions of all of the cytokines are limited in both magnitude and duration, and an understanding of the mechanisms by which their actions are negatively controlled is therefore essential. Control of cytokine signaling can occur at a number of regulatory points: the regulation of the production of a cytokine or its receptor; the regulation of the degradation of a cytokine or its receptor; or the control of the phosphorylation/dephosphorylation of substrates (including the cytokine receptors themselves, JAKs and other substrates).

Recent reports revealed a new family of proteins that are induced by cytokines but then inhibit cytokine action. The first member of this family was denoted as CIS, for cytokine-induced Src homology 2 (SH2)-containing protein [1]. The CIS gene was cloned originally as an immediate early gene that was induced by IL-2, IL-3 and erythropoietin (EPO). CIS was noted to associate with the tyrosine-phosphorylated EPO receptor (EPOR) and the IL-3 receptor β chain following stimulation with EPO or IL-3, respectively [1] (Figure 1). The presence of an SH2 domain in CIS could therefore explain the basis for this recruitment, although direct SH2–phosphotyrosine interactions between CIS and these receptors have yet to be demonstrated. From a functional perspective, it was noted that, when overexpressed, CIS could function as a negative regulator of IL-3-induced proliferation, although the mechanism of action of CIS was unclear [1]. One possibility is that CIS acts as an adaptor protein linking

Figure 1



Schematic of cytokine signaling, showing that CIS is regulated by STATs and is an inhibitor of STAT activation. CIS is shown associated with a receptor component, as it is known that CIS can associate with the EPO receptor and IL-3 receptor β chain, as discussed in the text. Two possible sites of action are shown for CIS. SOCS-1/JAB/SSI-1 (shown here as JAB out of space consideration) is shown to interact with JAKs and to inhibit their action.

cytokine receptors to other molecules involved in growth inhibition. This idea is based on the presence of an SH2 domain in the central portion of the molecule and amino-terminal and carboxy-terminal regions that are rich in proline and leucine residues that can be involved in mediating protein–protein interactions. Alternatively, it was also suggested that CIS might function by directly blocking phosphotyrosine motifs on receptors, preventing them from coupling to stimulatory signaling pathways [1]. Finally, given that CIS was observed to have a relatively short half-life, a third potential role for CIS was as a scavenger of tyrosine-phosphorylated proteins, targeting them for degradation [1].

Several observations have linked CIS to JAK–STAT pathways. First, induction of CIS in response to granulocyte-macrophage colony-stimulating factor (GM-CSF) and EPO is dependent on the presence of membrane-proximal regions of the common β chain, βc — a receptor chain that is a shared component of the receptors for IL-3, IL-5 and GM-CSF — and EPOR; these regions mediate JAK activation [1]. Second, the CIS promoter contains two pairs of tandem TTCNNNGAA motifs that are capable of binding Stat5, and a CIS-promoter reporter construct was induced by EPO in cells transfected with plasmids expressing EPOR and Stat5 [2]. In addition to the regulation of CIS production by the JAK–Stat5 pathway, it was interesting that CIS could negatively modulate Stat5 activation [2], suggesting that a possible negative-feedback loop operates. The JAK–STAT pathway does not seem to be the only pathway responsible for CIS induction, however, as CIS can be induced by tumor necrosis factor α (TNF-α) [3], a cytokine that is not known to activate JAKs or STATs.

Excitement over CIS increased tremendously earlier this year with the publication of three papers in *Nature* that reported the existence of CIS-related proteins. Remarkably, one of these proteins was cloned in three different labs by three entirely different experimental approaches. Starr *et al.* [3] cloned suppressor of cytokine signaling-1 (SOCS-1) as an inhibitor of IL-6-mediated differentiation and growth arrest of murine monocytic leukemic M1 cells; Endo *et al.* [4] cloned the same protein as a JAK-binding protein (JAB) that could interact with the catalytic (JH1) domain of Jak2; and Naka *et al.* [5] identified this protein using an antibody intended to be specific for SH2 domains of STATs — but which did not turn out to be completely specific — and found that it was not a STAT protein but instead was STAT-induced STAT inhibitor-1 (SSI-1). Collectively, the papers demonstrate that SOCS-1/JAB/SSI-1 is a potent inhibitor of IL-6 signaling, including IL-6-induced tyrosine phosphorylation of a component of its receptor, gp130, and Stat3. Interestingly, this CIS-related protein can interact not only with Jak2 but also with Jak1, Jak3 and Tyk2, and is an inhibitor of JAK catalytic activity (Figure 1) [4,5]. Consistent with its potential specificity for inhibiting JAKs, it did not inhibit fibroblast growth factor-mediated proximal events in NIH3T3 cells, such as receptor autophosphorylation, and phosphorylation of Shc and extracellular-signal-regulated kinase 2 (Erk2) [4].

Biological roles of CIS family proteins

A comparison of CIS, SOCS-1/JAB/SSI-1, and two other members of this family reveals a number of differences in their expression patterns. First, SOCS-1/JAB/SSI-1 is particularly highly expressed in thymus, and also is substantially expressed in spleen and lung, whereas CIS is more ubiquitously expressed [3]. Second, CIS can be induced by a wider range of cytokines than those that induce the other family members (Table 1) [3]: whereas CIS was originally noted to be induced by IL-2, IL-3 and EPO [1], it is now clear that CIS is also induced by IL-4, IL-7, IL-13, thrombopoietin (TPO), granulocyte colony-stimulating factor (G-CSF), GM-CSF, IFN-γ, TNF-α, IL-1, macrophage colony-stimulating factor (M-CSF), IL-6, IL-12 and leukemia inhibitory factor (LIF) [3]. In contrast, only a subset of these stimuli induce SOCS-1/JAB/SSI-1; for example, in bone marrow cells SOCS-1/JAB/SSI-1 is not induced by TNF-α, IL-1 and IL-7 [3]. Two additional members of the CIS family, SOCS-2 and SOCS-3, are somewhat more broadly expressed than SOCS-1/JAB/SSI-1 [3,6], with SOCS-3 showing the same pattern of induction as CIS [3].

On the basis of studies *in vitro* using cytokine-responsive cell lines, CIS and SOCS-1/JAB/SSI-1 appear to be negative regulators, as noted above (see Table 2 for a summary of known actions). First, forced overexpression of CIS in Ba/F3 cells results in decreased IL-3-induced proliferation [1], presumably as a result of the inhibition

Table 1

Cytokines that induce the CIS family members in bone marrow cells.

	CIS	SOCS-1/JAB/SSI-1	SOCS-2/SSI-2/CIS2	SOCS-3/SSI-3/CIS3
IL-1	+	–	+	+
IL-2	+	–	+/–	+
IL-3	++	+	+	+
IL-4	++	+	+	+
IL-6	+	–*	+/–	+
IL-7	+	–	+/–	+
IL-13	+	++	–	+
LIF	+/–	+/–	+	+
GM-CSF	++	+	+	+
G-CSF	+	–	+	++
M-CSF	+	–	–	++
EPO	+	+	++	+
TPO	+	–	+	+
IFN-γ	++	++	+	++

*Although IL-6 does not induce SOCS-1/JAB/SSI-1 in bone marrow cells, it induces this CIS family member in murine liver. Abbreviations as in the text.

Table 2

Known actions of the different CIS family members*.

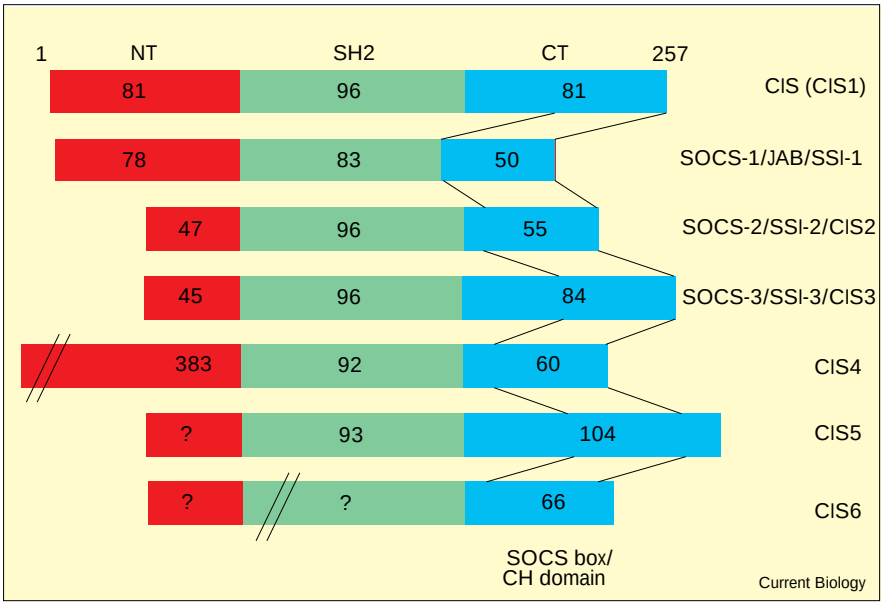
CIS family member	Action
CIS (now also denoted as CIS1):	Associates with IL-3Rβ and EPOR; negatively regulates IL-3-induced proliferation; inhibits STAT induction.
SOCS-1/JAB/SSI-1:	Associates with all four mammalian JAKs and inhibits their catalytic activity; inhibits IL-6-mediated differentiation and growth arrest of M1 cells; inhibits IL-6-induced phosphorylation of gp130 and Stat3; inhibits IL-2-mediated and IL-3-mediated c-*fos* promoter activity; inhibits EPO-mediated Stat5 activity.
SOCS-2/SSI-2/CIS2:	Inhibits LIF-mediated differentiation and growth arrest of M1 cells†; inhibits LIF-induced tyrosine phosphorylation of Stat3†.
SOCS-3/SSI-3/CIS3:	Associates with the JH1 domain of Jak2 and partially inhibits its catalytic activity; inhibits LIF-mediated differentiation and growth arrest of M1 cells; inhibits LIF-induced tyrosine phosphorylation of Stat3.
CIS4, CIS5, CIS6	None reported so far.

*Given the wide range of cytokines that activate different CIS family proteins and their broad tissue distribution, it is likely that broader ranges of action will be found for each of these molecules. †These results are as reported in [6], but there is disagreement about the ability of SOCS-2/SSI-2/CIS2 to exert these effects [7]. Abbreviations as in the text.

of transcriptional activity of several transcriptional targets of cytokine signaling. Second, CIS can inhibit Stat5-mediated signaling [2] and transcription from the c-*fos* promoter (M.J.A. and W.J.L., unpublished observations). Third, SOCS-1/JAB/SSI-1 completely abrogates the macrophage differentiation of the murine monocytic leukemic M1 cells in response to IL-6, LIF, oncostatin M and IFN-γ [3], and increases the proliferation of undifferentiated M1 cells [5]. This inhibitory effect seems to be specific to cytokine signaling as dexamethasone-induced differentiation is only partially inhibited and not totally abrogated by SOCS-1/JAB/SSI-1 [3]. Fourth, like CIS, SOCS-1/JAB/SSI-1 can inhibit transcription from the c-*fos* promoter [4] in addition to the activation of Stat3 [3]. The anti-proliferative activity of CIS and the inhibition of differentiation by JAB imply that dysregulation of these proteins may have a role in proliferative disorders and malignancies, particularly in the hematopoietic system. This hypothesis is consistent with the putative mapping of the human *CIS* gene to 3p21 (based on mapping of the murine *Cis* gene to chromosome 9), a region that is frequently deleted or rearranged in renal and bronchogenic malignancies (discussed in [1]).

SOCS-2 (also denoted as SSI-2 or CIS2) and SOCS-3 (also denoted as SSI-3 or CIS3) have been cloned in both murine [3] and human [6,7] systems. Moreover, three additional members of this family, denoted CIS4, CIS5

Figure 2



Schematic of CIS family proteins, depicting the lengths of the amino-terminal (NT), SH2, and carboxy-terminal (CT) domains. The SOCS box/CH domain is shown as part of the CT domain.

and CIS6, have been identified [7]. Whereas SOCS-1/JAB/SSI-1 is highly expressed in thymus, spleen, small intestine and peripheral blood leukocytes (PBL), SOCS-2/SSI-2/CIS2 is highly expressed in heart, placenta, lung, kidney and prostate and SOC-3/SSI-3/CIS3 is expressed in heart, placenta, lung, skeletal muscle and PBL. The differences in tissue distributions indicate that these CIS proteins might regulate different functions. Although both SOCS-2/SSI-2/CIS2 and SOC-3/SSI-3/CIS3 have been reported to inhibit LIF-induced tyrosine phosphorylation of Stat3 as well as LIF-induced growth arrest of M1 cells [6], some controversy remains regarding the actions of SOCS-2/SSI-2/CIS2 (see Table 2) [6,7]. The functions of CIS4, CIS5, and CIS6 remain unclear.

Functional domains of CIS family proteins

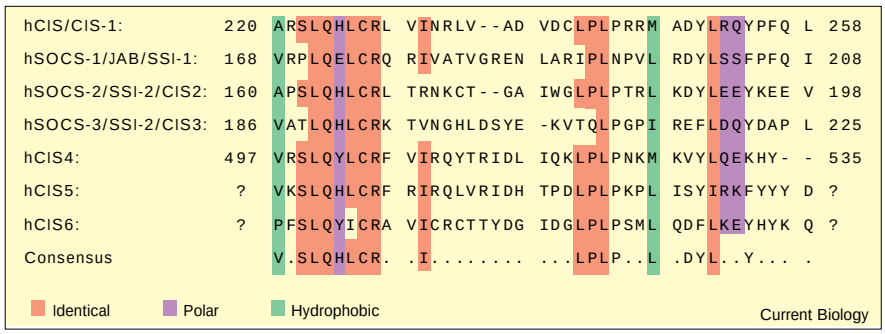
The functional domains of CIS proteins have been poorly studied. The isolated SH2 domain of SOCS-1/JAB/SSI-1) failed to inhibit autophosphorylation of Jak2 in 293 cells and had no inhibitory effect on the transcriptional activity of the c-*fos* promoter in Ba/F3 cells [4]. These data suggest a crucial role for the regions that are located amino-terminal or carboxy-terminal to the SH2 domain.

As noted above, both the amino-terminal and carboxy-terminal domains of CIS are rich in proline and leucine residues (10 Pro and 11 Leu in the amino-terminal 81 amino acids; 11 Pro and 12 Leu in the 80 carboxy-terminal amino acids), as are some of the other CIS family proteins. It is noteworthy that there is no significant homology between the family members in the amino-terminal region, suggesting either that this region is not functionally critical or instead that it might confer substrate specificity to the respective family member. Despite the lack of homology in the primary structure, however, the tertiary structures of the amino-terminal domains might be similar, although SOCS-2/SSI-2/CIS2 and SOCS-3/SSI-3/CIS3 have relatively short amino-terminal regions of approximately 45 amino acids, in contrast to the longer amino-terminal regions of CIS, SOCS-1/JAB/SSI-1, and CIS4 (Figure 2). Like the amino-terminal regions, the carboxy-terminal regions vary substantially in length, with human CIS (81 residues) and SOCS-3/SSI-3/CIS3 (84 residues) having longer carboxy-terminal regions, in contrast to the shorter regions of human SOCS-2/SSI-2/CIS2 (55 residues) and SOCS-1/JAB/SSI-1 (50 residues); nevertheless, the carboxy-terminal regions are more conserved than the amino-terminal regions. The carboxyl termini of the CIS family members contain a 40–50 amino-acid region of homology denoted as a SOCS box [3], or CH (CIS homology) domain (see Figure 3) [7]; however, the function of this region remains unknown. All the CIS family proteins share about 55% identity in their SH2 domains.

A number of major questions remain concerning this family of proteins. First, are all of these molecules inhibitory? Do any have both inhibitory and agonistic actions? Second, how do they exert their actions? CIS can interact with certain receptor molecules; for how many of these proteins is this the case? SOCS-1/SSI-1/JAB can interact with JAK family members: is this true for other members of the CIS family? Are these proteins adaptor molecules? If so, what types of proteins do they recruit? Given that some of these proteins can diminish phosphorylation, is this solely due to inhibitory effects on kinases or do these proteins recruit phosphatases as well? Do the CIS proteins block phosphotyrosine-docking sites for critical signaling pathways? Although possible, this seems less likely in view of the inability of the isolated SH2 domain of SOCS-1/JAB/SSI-1 to inhibit cytokine action [4]. Do they promote degradation of receptors or other molecules with which they might interact? What are the roles of the regions amino-terminal and carboxy-terminal to the SH2 domain? Third, what signaling pathways are inhibited? Clearly, there are data related to the JAK–STAT pathways and to the c-*fos* promoter for some of these proteins, but are other kinases and signaling pathways also affected? Fourth, how many more members of this family exist in addition to the seven that have been reported? Will there be as many or even more CIS family members as there are STATs? Also, which CIS family members are important for which cytokines?

Figure 3

Alignment of the SOCS boxes/CH domains of CIS family proteins.



In summary, an exciting new class of cytokine inhibitors that contain divergent amino-terminal regions, central SH2 domains and partially conserved carboxy-terminal regions has now been recognized. These proteins are rapidly induced by a number of cytokines and at least three members (CIS, SOCS-1/JAB/SSI-1 and SOCS-3/SSI-3/CIS3) have been shown to interfere with JAK–STAT signaling pathways. In addition to the need to further understand their mechanism(s) of action, it remains unclear to what degree these proteins have overlapping or distinct functions. The preparation of knockout mice in which each of these molecules is individually deleted, as well as knockout mice in which combinations of these proteins are deleted, should help to clarify the distinct functions of each of these proteins.

Nomenclature

A final word on nomenclature seems relevant. Currently, these proteins are being referred to using CIS, SOCS, JAB and SSI nomenclatures. Although all of these names have their own merit, the sooner agreement is reached upon this nomenclature issue, the easier it will be for the scientific community to understand the many studies that are likely to be published on this exciting family of molecules.

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